Correlation Between Activation of the Prelimbic Cortex, Basolateral Amygdala, and Agranular Insular Cortex During Taste Memory Formation

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Conditioned taste aversion (CTA) is a well-established learning paradigm, whereby animals associate tastes with subsequent visceral illness. The prelimbic cortex (PL) has been shown to be involved in the association of events separated by time. However, the nature of PL activity and its functional network in the whole brain during CTA learning remain unknown. Here, using awake functional magnetic resonance imaging and fiber tracking, we analyzed functional brain connectivity during the association of tastes and visceral illness. The blood oxygen level-dependent (BOLD) signal significantly increased in the PL after tastant and lithium chloride (LiCl) infusions. The BOLD signal in the PL significantly correlated with those in the amygdala and agranular insular cortex (IC), which we found were also structurally connected to the PL by fiber tracking. To precisely examine these data, we then performed double immunofluorescence staining with a neuronal activity marker (c-Fos) and an inhibitory neuron marker (GAD67) combined with a fluorescent retrograde tracer in the PL. During CTA learning, we found an increase in the activity of excitatory neurons in the basolateral amygdala (BLA) or agranular IC that project to the PL. Taken together, these findings clearly identify a role of synchronized PL, agranular IC, and BLA activity in CTA learning.

Keywords: basolateral amygdala, fMRI, learning and memory, prelimbic cortex, taste aversion

Introduction

Gustatory memory plays an important role in food selection. The recognition of tastes is frequently an instinctive response, part of an innate behavioral repertoire, but it usually includes brain processes involved in recalling past experiences when the taste stimulus was associated with consequences of its ingestion. Conditioned taste aversion (CTA) is a commonly used model to investigate the process by which animals learn to associate a taste (conditioned stimulus; CS) with visceral malaise induced by a drug treatment after a trace interval (unconditioned stimulus; US). Consequently, animals show a changed palatability of the taste stimulus without showing a changed perception of the taste. CTA is formed by single-trial training and is a long-lasting memory that provides a useful model for studying the different phases of memory including acquisition.

It is well known that the rostral part of the agranular insular cortex (IC) and the basolateral amygdala (BLA) is crucially important for both acquisition and expression of CTAs (Gallo et al. 1992; Schafe and Bernstein 1998; Reilly and Bornova-lova 2005). It has also been documented that the other brain regions are related to taste aversion learning (Bermudez-Rattoni 2004). The medial prefrontal cortex (mPFC) is involved in learning, guiding behavior, executive functions, and decision making (Arnsten and Li 2005). Most of the data involving the mPFC in taste learning refer to CTA extinction. The c-Fos expression in both the prelimbic (PL) cortex and infralimbic (IL) cortex in the mPFC increases after complete extinction of CTA (Mickley et al. 2005). Protein synthesis inhibition in the mPFC impairs CTA extinction (Akirav et al. 2006). In addition, some studies have shown the involvement of the mPFC in CTA acquisition. Lesions within the mPFC impair CTA learning (Hermadi et al. 2000), and cutting corticocortical connections impairs performance of CTA (Schalmon et al. 1994). The blockade of mPFC β-adrenergic receptors before acquisition also impairs aversive taste memory (Reyes-Lopez et al. 2010). However, the functional connectivity of the mPFC with other regions during CTA acquisition remains elusive.

In this study, we combined a functional magnetic resonance imaging (fMRI), fiber tracking, and immunofluorescence to qualitatively and quantitatively delineate the forebrain activities and the network in the process of CS-US convergence. We focused on the PL in the mPFC, because it has been shown that the PL is important for the acquisition of trace conditioning (Gilmartin and Helmstetter 2010; Gilmartin et al. 2013), where the CS and US do not overlap. Our goal was to provide (1) the PL activity and network during CTA learning and (2) precise structural analyses of fMRI-based functional connectivity and fiber tracking data by using immunofluorescence. fMRI in awake animals has provided a valuable opportunity to investigate whole-brain responses and connectivity among various brain regions with temporal resolution. Furthermore, using fiber tracking, we investigated a structural connectivity among the brain regions that was extracted from the functional connectivity study. Fiber tracking, which can be calculated from diffusion tensor imaging (DTI), provides a global delineating structural connectivity of the axon fibers based on the movement of water molecules (LeBlanc and Ducharme 2007). Although the combined fiber tracking and fMRI study is a novel and strong noninvasive technique to understand the global network in the whole brain (Elhofer et al. 2011; Marenco et al. 2012), there are some limitations of understanding the microstructure; (1) the blood oxygen level-dependent (BOLD) increase could be the amount of activations of excitatory neurons, inhibitory neurons, and astrocytes (Logothetis 2008). (2) Functional connectivity and fiber tracking analyses could show direct and/or indirect brain connections without directions. To overcome these limitations, we performed 2 immunohistochemical experiments. We first examined neural activation in the regions where we observed a significant
BOLD increase by expression of c-Fos, which is synthesized during certain forms of neuronal activation. We then investigated the PL afferent neural activation by using c-Fos expression coupled with retrograde transport of the tracer cholera toxin-b (CTb) from the PL.

Materials and Methods

Subjects and Treatments
All experimental procedures were approved by the Institutional Animal Care and Use Committee of Ajinomoto Co., Inc. and confirmed to the standards for the use of laboratory animals published by the Institute of Laboratory Animal Resources, US National Academy of Sciences.

Male Wistar rats (6 weeks of age, weighing 190–210 g) were obtained from Charles River Japan (Yokohama, Japan). All rats were pair-housed in plexiglass cages until surgery. The vivarium was maintained at a constant temperature and humidity (23 ± 1°C, 50–60%) with a 12:12 light/dark cycle (lights on 7 AM to 7 PM). Food and water were provided ad libitum. All animals were handled for 5 min/day over 3 days before the surgery.

Surgery
The surgeries for intraoral (IO) cannulae and MRI fixation have been described elsewhere (Tsurugizawa et al. 2010; Uematsu et al. 2011). Briefly, rats were anesthetized by intraperitoneal (IP) injection of sodium pentobarbital (50 mg/kg), placed in a stereotaxic device, and laid on a thermo pad at 37°C until recovery from anesthesia. An incision was made on the midline of the scalp. A beveled 19-gauge needle was placed at a point slightly lateral to the first maxillary molar on either side of the mouth and advanced subcutaneously to the temporal muscle near the top of the skull surface. The needle was then replaced with a PE-90 catheter (Natsume Seikakusho Co. Ltd., Tokyo, Japan). The IO end of the cannula was heat-flared to an approximate diameter of 5 mm to prevent it from being drawn into the oral mucosa. The other end was fixed to the skull with super bond C&B (Sunmedical Co. Ltd., Shiga, Japan) and dental cement (GC Corp., Tokyo, Japan). For head fixation during MRI, cranioelastic acrylic cement was applied in 2 holes molded on each side to serve as a receptacle for fiberglass bars. The oral cannulae did not interfere with the eating behavior of the animals. All rats were allowed 7 days of recovery after the surgery, and their cannulae were flushed by deionized water every other day.

MRI Acclimation
The procedure for scanning awake rats has been described in detail previously (Tsurugizawa et al. 2010). Briefly, we trained the rats over 5 days. In the first 3 days, we used a mock MRI system consisting of a nonmagnetic bore and head positioner. Rats were lightly anesthetized with 2% isoflurane. The head was fixed to the skull with super bond C&B (Sunmedical Co. Ltd., Japan), dental cement, and acrylic-mounted head was then fixed with 4 fiberglass bars, held in place with acrylic cement, and the body was gently restrained with elastic bands. To reduce stress due to noise, ear plugs were used. The rats were habituated to the scanner environment and trained to remain motionless during the trials. In the next 2 days, we trained the rats in an MRI system using the same conditions as the fMRI measurement conditions described below, except without administering an infusion.

MRI Acquisition
All fMRI measurements were performed during the light period. The rats were lightly anesthetized with 2% isoflurane, and a 2% lidocaine paste was applied around the abdominal skin to reduce discomfort caused by the insertion of the needle for IP injection. The cranioelastic acrylic-mounted head was then fixed within 4 fiberglass bars on a nonmagnetic stereotaxic apparatus. When the rat was fully conscious, the stereotaxic apparatus was placed inside the MRI bore. Injections were made via a plastic syringe connected at the end of the IO cannula tubing and IP needle. Cannula placement was verified after imaging.

The MRI system was a Bruker Avance III system (Ettlingen, Germany) with a 4.7-T/40-cm horizontal superconducting magnet and gradient coils (26/12-cm diameter). BOLD fMRI data were obtained using a $T_2$*-weighted multislice fast low-angle shot sequence with the following parameters: time of repetition = 15 s, echo time = 10 ms, flip angle = 30°, field of view = 35 × 35 mm$^2$, acquisition matrix = 64 × 64, slice thickness = 1.3 mm, and slice number = 17. Anatomical images were obtained by a multislice rapid acquisition with relaxation enhancement (RARE) sequence in the same orientation as the functional sequences using the following parameters: time of repetition = 2500 ms, effective echo time = 60 ms, RARE factor = 8, acquisition matrix = 128 × 128, field of view = 35 × 35 mm$^2$, slice thickness = 1.3 mm, slice number = 17, and number of averages = 4.

Experimental Protocol
The functional scanning was continuously conducted for 50 min. Ten minutes after the start of scanning, a saccharin solution was delivered into the mouth through the implanted IO cannula for 10 min at a rate of 200 μL/min and then lithium chloride (LiCl) (CS–US, N = 8) or saline (CS–saline, N = 8) was infused into the abdomen through the IP needle for 15 s at a rate of 40 mL/min/kg body weight using a syringe pump (CVF-3200; Nihon Kohden, Tokyo, Japan). For the US group, 20 min after the start of scanning, LiCl (N = 8) was delivered into the abdomen at a same dose and rate as above. During scanning, respiration rate, heart rate, and body temperature were monitored with a magnetic resonance-compatible monitoring system (model 1025; SA Instruments, Stony Brook, NY, USA). We choose 0.2% saccharin diluted with deionized water as the CS. We used 150 mM LiCl (Wako, Tokyo, Japan) at the US in the CS–US group and saline in the CS–saline/saline group. In separate sessions from the fMRI experiments, DTI images were obtained for fiber tracking. During the DTI sessions, anesthesia was maintained with 1.5% isoflurane in air. The cranioelastic acrylic-mounted head was fixed and the body temperature was maintained at 36°C. DTI data were acquired with a 4-segmented, spin echo multishot echo planar imaging pulse sequence with following parameters: time of repetition = 12.5 s, echo time = 23.84 ms, field of view = 21 × 21 mm$^2$, matrix = 70 × 70 (in-plane resolution = 300 μm), 15 consecutive slices, slice thickness = 300 μm, Δ/δ = 5/9.5 ms, and 10 averages. The receiver bandwidth was 200 kHz. Symmetric diffusion gradients were applied with a b = 1000 s/mm$^2$ in 30 non-collinear directions.

fMRI Analysis
Data were preprocessed using the SPM5 software (Welcome Trust Center for Neuroimaging, London, UK) in MATLAB (MathWorks, Inc., Tokyo, Japan). All functional images were realigned to the first image, and then coregistered to the anatomical image. Anatomical and functional images were then normalized to the Paxinos and Watson rat brain atlas (Paxinos and Watson 2007). Normalized functional images were smoothed with a 0.8-mm full-width at half-maximum isotropic Gaussian kernel. Statistical analyses were conducted using a custom program written in MATLAB. We determined the brain regions demonstrating significant BOLD changes by applying boxcar functions (Tsurugizawa et al. 2009; Uematsu et al. 2010). The “OFF” period was the period 5 min before IO infusion (basal period: −15 to −10 min), and the “ON” periods were every 5 min after the end of the IO infusion for a total of 6 periods: 0–5, 5–10, 10–15, 15–20, 20–25, and 25–30 min. The images during IO injection were discarded because of motion artifacts caused by jaw movements. If any image sets contain motion artifacts after IP injection, they were also discarded. The first-level analysis (fixed-effect analysis) was performed using the data from individual rats. To make inferences about group data, second-level analyses (random-effect analyses) were conducted between and within the groups. The t-maps were set at a threshold of P < 0.05 corrected for multiple comparisons using a false discovery rate procedure, with a minimum cluster size of 32 voxels. We confirmed that there were no significant changes in BOLD signal intensity during each ON period when rats were left undisturbed. All functional data were overlaid on the template in MRicron for presentation purposes.
FMRI Connectivity Network Analysis

Regions of interest (ROIs) were created in MIRCron. We drew the ROIs in the amygdala, rostral IC, and PL by referring to the significant BOLD signal increase seen on the fMRI results \( (P < 0.05) \) (Cordes et al. 2000). These ROIs were used to obtain time courses in each animal. The time course of changes in BOLD signals within each ROI was calculated as follows:

Percent change in BOLD signal intensity (\( \% \)) = \( \left( \frac{BOLD \ signal \ intensity \ within \ an \ ROI \ during \ an \ “ON” \ period/averaged \ BOLD \ signal \ intensity \ within \ ROI \ in \ basal \ period \right) - 1 \right \) \times 100.

Maps of BOLD signal changes and correlations across subjects were determined by comparing the BOLD signal changes in the ROIs and calculated using a program written in MATLAB. For first-level analysis, we constructed a design matrix comprising a regressor that captured the group mean signal within each ROI. Second-level analysis was performed across the subjects. The areas showing a significant BOLD increase \( (P < 0.05, \ corrected \ for \ multiple \ comparisons \ using \ a \ false \ discovery \ rate \ procedure) \) and those with a cluster size >32 voxels were included in the T-contrast images.

Fiber Tracking

Fiber tracking using the DTI data was analyzed using the DSI studio software (http://dsi-studio.labsolver.org). To initiate the fiber tracking, we created the ROIs of the PL, amygdala, IC, and bed nucleus of the stria terminus, using a T2-weighted anatomical image that was coregistered to the DTI images in DSI studio. These ROIs were used as the ROI that fibers pass in DTI tractography. Tactography was carried out based on Fiber Assignment by Continuous Tracking algorithm with fiber propagation starting at a fractional anisotropy (FA) threshold value of 0.2 and a turning angle threshold value of 65°. The fiber propagation was stopped at FA value <0.2 and turning angle <65°. We initially performed whole-brain fiber tracking with the ROI of the PL to draw the all fibers that passed the PL. Then, we extracted the fibers that passed 2 ROIs, for instance, the PL and the amygdala, or the PL and the IC.

Behavioral Analysis

The procedure used for the taste reactivity test has been described elsewhere (Berridge 2000). Briefly, rats were placed in a transparent chamber for 10 min where they received an IO infusion of saccharin solution (0.5 mL/min) for the last 2 min. A mirror located beneath the chamber at a 45° angle reflected a ventral view of the rat and allowed us to record mouth movements using a digital video camera. Taste reactivity was assessed by an investigator blinded to the stimulus conditions using the Observer XT event-recording program (Noldus Information Technology, Wageningen, the Netherlands). The hedonic score included the sum total frequencies of single tongue protrusions, 2-s bouts of rhythmic tongue protrusions, and 5-s bouts of paw licking. Tongue protrusions included midline and lateral extensions of the tongue. Averse responses included the sum total frequencies of gaping, chin-rubbing, head shaking, and forelimb flailing. The observational data from the CS–US and CS–saline groups were compared using the Student’s t-test.

CTB Injection

Rats examined for retrograde axonal tracing (CS–US; \( N = 4 \), CS–saline; \( N = 5 \), and LiCl; \( N = 5 \)) with immunohistochemistry were placed in the stereotaxic apparatus under isoflurane anesthesia (5% for induction and 2% for maintenance). After adjusting the flat skull position, a glass micropipette (20–30 µm tip diameter) was directed toward the PL (+3.7 mm anteroposterior; +0.5 mm mediolateral; −3.5 mm dorsoventral from bregma) (Paxinos and Watson 2007). CTB (C-22843; Life Technologies Japan, Tokyo, Japan) diluted to 0.5% (wt/vol) with 0.1 M phosphate buffer saline (PBS), pH 7.3, was unilaterally infused with air pressure (20 psi, 10 ms, and 10 bursts; Pressure System IIe, Tooyee Company, Fairfield, NJ, USA) and the glass micropipette left in place for 5 min after the infusion to allow for diffusion and to reduce spread up the injection tract.

One week after surgery, animals were subjected to a behavioral experiment in which they received IO saccharin and/or IP injection under the same conditions as in the MRI experiments. Two hours after IP injection, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, IP) and transcardially perfused with 100 mL of 0.9% saline, containing heparin (5000 iu/mL), followed by 400 mL of 4% paraformaldehyde in 0.1 M PBS. Their brains were postfixed in the same fixative overnight and placed in a 30% sucrose solution for 48 h. Brains were blocked using a matrix aligned to the Paxinos and Watson rat brain atlas (Paxinos and Watson 2007), and 40-µm coronal sections were cut using a cryostat (Leica Microsystems, Tokyo, Japan).

Immunofluorescence

For immunofluorescence, free-floating sections were washed repeatedly in 0.1 M PBS containing 0.3% Triton-X (PBST), followed by a 1-h incubation in PBST containing 2.0% normal donkey serum (PBSTBS). Sections were then incubated in the primary antibodies diluted in PBSTBS for 24 h at 4°C in the dark. The primary antibodies were a rabbit anti c-Fos antibody (1: 5000; PC38, Millipore, Tokyo, Japan) and a mouse anti-GAD67 antibody (1: 2000; MAB5406, Millipore). After washing with PBST, sections were then incubated for 1 h at room temperature in secondary antibodies diluted in PBSTBS. The secondary antibodies were Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 647 donkey anti-mouse (1: 1000, Life Technologies Japan). After washing with PBST, sections were mounted onto slides and coverslipped with Fluormount (Cosmobicio Co., Ltd, Tokyo, Japan). For the c-Fos study, we used the same protocol except anti-GAD67 antibody and Alexa Fluor 647 donkey anti-mouse antibody.

Counts of immunoreactive (IR) neurons for c-Fos, GAD67, CTB, and double-labeled c-Fos/CTB were examined under a confocal laser scanning microscope (Fluoview, Olympus) by an observer blinded to the group allocations. All sections counted were 120 µm apart. The amygdala was assessed over 6 sections beginning at bregma from −2.72 to −2.0 mm. The IC was assessed over 6 sections beginning at bregma from +0.7 to +1.42 mm. The central nucleus of the amygdala (CeA) and the BLA were defined as areas bordered by GAD67-positive neurons. The agranular and dysgranular IC were defined as areas that bordered by the rhinal fissure and the presence of 4 layered cortex. The number of positive cells were averaged in each region and used following analyses. The total counts of single-labeled c-Fos- and CTB-reactive neurons, as well as dual-labeled c-Fos/CTB neurons in the BLA and IC, were evaluated using the analysis of variance (ANOVA), followed by Tukey–Kramer post hoc tests.

Results

Acquisition of Aversive Response to the CS in Rats Given a US

To observe neural responses during acquisition of the CS–US association, we performed fMRI in awake animals. Rats inside the MRI bore received an IO infusion of 0.2% (w/v) saccharin for 10 min, immediately followed by an IP infusion of 150 mM LiCl or saline. To study the extent to which rats acquire taste aversion, rats were subjected to taste reactivity tests 2 days later. In this test, rats received an IO infusion of 0.2% saccharin solution for 2 min and were observed in a pleioglass box by a ventral view to assess the hedonic and aversive responses to saccharin. The CS–US group displayed a robust aversion to saccharin, whereas the CS–saline group showed a higher hedonic response to saccharin (Fig. 1). Therefore, only the CS–US group acquired taste aversions to the saccharin solution.

BOLD Responses in Forebrain Regions in the CS–US, CS–Saline, and US Groups

We analyzed the BOLD signal changes after the IP injection of LiCl, which were related to CTA learning. We omitted BOLD signal analyses during IO infusion because of the consistent
presence of motion artifacts related to jaw and tongue movements. Images were compared every 5 min after the end of the IP infusion with the baseline images obtained at −15 to −10 min (Fig. 3A,B). Though the respiration rate did not change in each period, heart rate significantly decreased after IP LiCl injection compared with the baseline period in the CS–US and US groups (Fig. 2). The pertinent rat brain atlas sections overlaid with the corresponding fMRI data are shown in Figure 3C. The spatial parameter maps of BOLD responses are shown in Figure 3D–F. In the CS–US group, the BOLD signal intensity increased significantly in the bilateral amygdala, bed nucleus of the stria terminalis (BNST), PL, hippocampus, orbitofrontal cortex (OFC), ventromedial hypothalamus (VMH), ventral posterior area of thalamic nucleus (VPTthN), and left rostral IC. In the CS–saline group, there were no significant increases in BOLD signals in any region of the brain throughout the scanning period. In the US group, the BOLD signal increased significantly in the bilateral amygdala, BNST, VPTthN, and left hippocampus. In the saline group, there were no significant BOLD increases throughout the scan period. The t-values and the periods when the BOLD signals changed most markedly in each brain region are given in Table 1.

We then analyzed between-group comparisons. Compared with the CS–saline group, the CS–US group showed significantly higher BOLD signals in the bilateral amygdala, rostral IC, BNST, hippocampus, PL, VPTthN, left OFC, and left VMH. Compared with the US group, the CS–US group had significantly higher BOLD signals in the bilateral PL, left amygdala, left rostral IC, left hippocampus, right OFC, and right VPTthN (Supplementary Fig. 1).

We next performed c-Fos immunohistochemistry to confirm neural activity in the PL, BLA, and IC. The fixation time was determined to be 2 h after IP injection in a previous report (Koh and Bernstein 2005). The number of c-Fos IR neurons in the CS–US group was significantly higher than in the BLA, agranular IC, and PL, compared with the other groups (Fig. 4). In dysgranular IC, the CS–US and US groups exhibited a significantly higher number of c-Fos IR neurons than the CS–saline and saline groups.

**Functional Connectivity in the Amygdala, IC, and mPFC During the Formation of CS–US Associations**

We examined ROIs from one hemisphere based on a report suggesting lateralization of function in several nuclei during CTA acquisition (Clark and Bernstein 2009). We analyzed the left hemisphere because it showed more significant increases of BOLD signals than the right hemisphere in CS–US group (Fig. 5A). The BOLD signal time courses from the ROIs were individually calculated and used as a regressor to identify brain regions, in which BOLD signal fluctuations were highly correlated with the ROI.

The time course of mean BOLD signal fluctuations from the ROIs in the CS–US group is shown in Figure 5B, and the coronal view of the functional connectivity maps is presented in Figure 5C. BOLD signal changes in the left PL correlated strongly with those in the left IC, left amygdala, and right BNST. The coronal view of the functional connectivity maps in the CS–saline and US groups is presented in Figure 5D,E, respectively. In the CS–saline group, there were no significant correlations among the amygdala, IC, and PL.

**Structural Connectivity in the Amygdala, IC, and PL**

The first tracking, presented in Figure 5F, showed abundant fibers projecting over a wide range from the PL. Fiber bundles were oriented to the anterior/posterior cortex, corpus callosum, limbic system, and hypothalamus. Structural connectivity between the ROIs was analyzed using the PL, amygdala, IC, and BNST (Fig. 5F). Fiber bundles between the amygdala and PL passed through the corpus callosum (Fig. 5G). Fiber bundles connecting the amygdala and IC and the PL and IC had 2 pathways (dorsal and ventral curvatures) (Fig. 5H). The other regions, in which BOLD signals were increased by CTA
Figure 3. Illustration of the fMRI protocol used in the (A) CS–US and CS–saline groups and (B) US group. Functional data were obtained every 15 s for 50 min. A boxcar function generated an "OFF" period 5 min before IO administration of saccharin (blue bar) and an "ON" period every 5 min after the start of IP administration (red bars). (C) Relevant brain coronal sections from the rat brain atlas used in this study. Coronal sections of several brain regions in the (D) CS–US, (E) CS–saline, and (F) US groups during the 0–5- and 15–20-min periods. T-maps depicting the areas exhibiting a significant increase in BOLD signal intensity compared with the "OFF" period. The color bar represents the t-value. AMY, amygdala.
Analysis revealed dense CTb-positive cells in the ipsilateral noreactivity in the IC and BLA for c-Fos, CTb, and GAD67.

Distribution of c-Fos and GAD67 Immunoreactivity in PL Afferents

To further examine the activation of BLA and IC cells projecting to the PL, we then performed c-Fos/GAD67 immunohistochemistry combined with retrograde tracing. Expression of c-Fos is used as a marker of cell activation, and GAD67 is used as a marker of inhibitory neuron. The spread of CTb was confirmed with fluorescence. Any rats with CTb injections lateral or ventral from the PL were excluded from the analysis. Figure 6A,B shows a representative photomicrograph of immunoreactivity in the IC and BLA for c-Fos, CTb, and GAD67. Analysis revealed dense CTb-positive cells in the ipsilateral agranular IC and BLA. In the IC, there were no differences in the number of CTb-positive neurons among the groups ($F_1, \gamma = 1.0, P>0.1$). We found no CTb and GAD67 dual-positive cells in BLA and IC. There were significant differences in ratio of c-Fos and CTb dual-positive neurons/CTb-positive neurons among the groups ($F_1, \gamma = 5.1, P<0.05$). Post hoc analysis revealed that the ratio of dual-positive cells in the CS–US group was significant and 3-fold higher than those of the CS–saline or US groups (Fig. 6C). Figure 6D,E shows representative photomicrographs of immunoreactivity in the BLA for c-Fos and CTb. There were no differences in the number of CTb-positive neurons among the groups ($F_1, \gamma = 0.12, P>0.1$). In the BLA, there were significant differences in c-Fos and CTb dual-positive neurons/CTb-positive neurons among the groups ($F_1, \gamma = 29.7, P<0.01$). Post hoc analysis revealed a significant and 4-fold higher ratio of dual-positive cells in the CS–US group compared with the CS–saline or US groups (Fig. 6F).

Discussion

In this report, we used the combined awake fMRI, DTI, and immunohistochemistry to demonstrate a forebrain neural network engaged during the formation of associations between a taste and visceral malaise. We found that the PL, amygdala, and rostral IC showed significant BOLD increases during CS–US association. The functional connectivity study clearly extracted a neural network involving the PL, amygdala, and IC during CTA learning. Interestingly, the BNST, OFC, and hippocampus were not involved in this connectivity, although BOLD signals in these regions were also significantly increased during CTA learning. The DTI analyses showed that the PL is structurally connected to the amygdala and rostral IC. Furthermore, we observed a significant increase in c-Fos IR in afferent excitatory neurons in the both BLA and rostral part of agranular IC during the formation of associations between a taste and visceral malaise.

For taste processing, peripheral nerves first project into the rostral part of the nucleus of the solitary tract, and then send ipsilateral projections to the dorsolateral parabrachial nucleus. Projections from the dorsolateral parabrachial nucleus reach the lateral hypothalamus, BNST, CeA, BLA, and medial area of the VPThN. In the CS–saline group, these nuclei were not activated during the first period, indicating that there were no remaining gustatory perceptions after the IO infusion ended. In addition, the CS–saline group did not show any BOLD increases in most regions during any of time periods examined; thus, ingested saccharin did not affect BOLD signals.

During CTA, visceral response information is transmitted to the caudal part of the solitary tract nucleus and then to the lateral parabrachial nucleus, CeA, and lateral area of VPThN (Bermudez-Rattoni 2004). Previous studies have shown that LiCl administration induces a robust increase in c-Fos expression in the CeA, BLA, BNST, and VPThN, but not in the IC (St Andre et al. 2007). Similarly, our study showed increased BOLD signals upon LiCl treatment in the amygdala, VPThN, and BNST, but not in the IC. The c-Fos IR cells were increased in the CeA, which is composed of inhibitory neurons, in the US group as well as in the CS–US group. These results indicate that the BOLD signal increase reflects the CeA activation by LiCl treatment. The change in heart rate after IP infusion of LiCl is consistent with previous studies (Kosten and Contreras 1989; Abdel-Zaher and Abdel-Rahman 1999). As BOLD signal

Table 1

<table>
<thead>
<tr>
<th>Brain regions showing the most significant increases in the BOLD signal</th>
<th>CS-US</th>
<th>CS-saline</th>
<th>US</th>
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<tbody>
<tr>
<td>noreactivity</td>
<td>t-value</td>
<td>Period</td>
<td>t-value</td>
</tr>
<tr>
<td>Amy</td>
<td>12.3/9.9</td>
<td>3/3</td>
<td>10.5/8.5</td>
</tr>
<tr>
<td>BNST</td>
<td>9.8/7.5</td>
<td>3/3</td>
<td>8.9/3.3</td>
</tr>
<tr>
<td>IC</td>
<td>11.5/7.5</td>
<td>3/3</td>
<td>8.1/3.3</td>
</tr>
<tr>
<td>Nac</td>
<td>11.5/10.2</td>
<td>6/6</td>
<td>11.7/–</td>
</tr>
<tr>
<td>OFC</td>
<td>11.7/7.7</td>
<td>3/4</td>
<td>11.7/3</td>
</tr>
<tr>
<td>PL</td>
<td>11.7/10.5</td>
<td>3/4</td>
<td>11.7/–</td>
</tr>
<tr>
<td>VMH</td>
<td>7.5/8.1</td>
<td>3/6</td>
<td>7.2/9.1</td>
</tr>
<tr>
<td>VPThN</td>
<td>12.1/7.2</td>
<td>6/5</td>
<td>6.7/8.6</td>
</tr>
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</table>

Note: In the period column, 1–6 represent 0–5, 5–10, 10–15, 15–20, 20–25, and 25–30 min after IP injection, respectively.

*, not significant.
relies on blood flow and blood volume, the question of whether changes in heart rate affected BOLD signals after LiCl injection arises. However, c-Fos expression mapped the BOLD response in the BLA, rostral IC, and PL, indicating that the BOLD signal increase after LiCl infusion was not a heart rate-derived artifact.

It is clearly shown that CS–US information converges on individual BLA and gustatory IC neurons during CTA acquisition by cellular compartmental analysis of temporal gene transcription by fluorescence in situ hybridization with Arc, which is an immediately early gene (Barot et al. 2008). Consistently, the present study demonstrated significant increases in BOLD signals in the amygdala and IC in the CS–US group. In addition, these nuclei showed higher BOLD signals than those in the US group. The immunohistochemical experiments confirmed that the c-Fos-positive cells were significantly increased in the BLA and in the agranular IC. In accordance with our data, more BLA neuronal activation is seen in response to US when it followed a novel CS than when it occurred alone or when it followed a familiar taste (Barot et al. 2008). This pattern is identical in the CS–US convergence during fear conditioning (Barot et al. 2009), indicating the consistency of the CS-dependent processing of aversive information in BLA neurons.

Anatomically, the BLA and IC are reciprocally connected (Reep and Winans 1982a, 1982b). By recording the neural response to saccharin as a taste stimulation before and after CTA, it has been demonstrated that the neuronal coherent activities between the gustatory cortex and BLA are increased after learning (Grossman et al. 2008). The tetanic stimulation of BLA induced long-term potentiation in the ipsilateral IC by N-methyl-D-aspartate (NMDA) receptor-dependent way (Escolar et al. 1998; Jones et al. 1999). CTA training produced persistent changes in the induction of subsequent IC long-term potentiation by BLA tetanic stimulation (Rodriguez-Duran et al. 2011). Taken together, the plasticity of the BLA and IC could occur during CS–US associations. Consistent with this hypothesis, our study showed functional connectivity of the IC and amygdala (Supplementary Fig. 2), indicating that the neural associations between these nuclei occur after LiCl injection.

The functional connections of the PL with other brain regions during CTA remain uncertain (Nunez-Jaramillo et al. 2010). Here, we observed that the activities of the amygdala and IC are both highly correlated with activity in the PL. From the fiber tracking, we confirmed that the PL is structurally connected with the amygdala and IC in accordance with previous reports (Heidbreder and Groenewegen 2003; Vertes 2006; Hoover and Vertes 2007). However, there are some limitations to fMRI and fiber tracking analysis. Spatial resolution is about 300 µm for DTI and 500 µm for fMRI. Also, fiber tracking is sensitive to indirect and direct connections. To overcome these problems, we performed c-Fos immunofluorescence with CTb injection into the PL. We found that (1) the PL receives input from BLA or rostral IC and (2) the CS–US group exhibited 3- to 4-fold higher expression of c-Fos-positive and CTb double-positive cells than the other groups, consistent with our functional and structural connectivity analyses. Importantly, there was no functional connectivity between the PL and BNST, even though fiber tracking showed PL and BNST structural connections. Anatomical studies show that the PL and BNST are not connected directly (Gannon and Millan 2007; Zhao et al. 2007), suggesting that these fiber tracking data reflect indirect connections.
connections that are not involved in the PL circuit during CTA learning. We also found many functionally connected areas with the PL in the CS–saline group. However, almost all of those regions did not show a significant BOLD increase in fMRI analysis, suggesting that this result reflects resting-state functional connectivity in normal state. In support of this view, the PL is correlated with the hippocampus and cingulate cortex, but not with the amygdala, during resting state (Lu et al. 2012). With respect to the PL neural circuits of learning, some BLA neurons that send projection to the PL are activated by the CS after fear learning (Herry et al. 2008). Furthermore, inactivation of the BLA in conditioned rats resulted in a decreased firing rate of putative PL pyramidal neurons to the CS (Silvagni et al. 2008). In fear conditioning, the PL is critical for the expression of conditioned fear, and the response of PL neurons to a CS is higher after conditioning. A CS presentation after conditioning elicits the activation of BLA neurons, which send outputs to the mPFC, but receive no input from the mPFC (Herry et al. 2008). Consistent with this view, our CTA experiments show that BLA excitatory neurons, which project to PL, are activated by CS–US presentation. A notable finding in this study is the connection between the PL and IC. To our knowledge, there is little previous evidence for these functional connections.

We also found the significant BOLD signal increase of the OFC and hippocampus in the CS–US group. The brain imaging studies have described a particular role of the OFC in...
emotional processing in humans and primates (Murphy et al. 2003; Fox et al. 2010). Additionally, a previous fMRI study showed that the OFC is activated in both the early and late phases of the fear-conditioning process (Tabbert et al. 2005). The OFC is involved in top-down processing, implying that the OFC modulates neural responses in other brain structures during CTA acquisition. However, it is shown that lesions of OFC do not affect the CTA acquisition (Gallagher et al. 1999).

As for the hippocampus, lesions of the hippocampus impairs long-trace CTA, where a long interval (3 h) is interposed between taste exposure and induction of illness, but it does not affect standard CTA acquisition (Koh et al. 2009), indicating that the BOLD signal increase in hippocampus is not important for CTA acquisition. Interestingly, our functional connectivity experiments showed that BOLD signals in the OFC or hippocampus were not significantly synchronized with those in the PL, BLA, and IC, despite its activation during CTA learning. Therefore, the OFC or hippocampus may oppose the actions of those brain regions and might have another role in this paradigm.

In conclusion, our study adds important evidence elucidating the neurobiology of CTA. Although the role of the mPFC in CTA extinction has been recognized (Mickley et al. 2005; Akirav et al. 2006; Maroun et al. 2012), our results shed light on the PL during the acquisition of taste preferences. Furthermore, our data demonstrate that the mPFC, especially the PL, is functionally connected with both the BLA and agranular IC. These interactions are potently increased during the acquisition and could maintain the association of taste with its consequences. Future work will continue to expand our understanding of the multiarea interactions and clarify these functions during the formation of aversive memory.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

Notes
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References


